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Comparison of a Rapid Culture Method Combining an Immunoperoxidase Test and a Group Specific Anti-VP1 Monoclonal Antibody With Conventional Virus Isolation Techniques for Routine Detection of Enteroviruses in Stools

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In order to shorten the time required for the detection of enteroviruses in stool specimens, an 18-h immunoperoxidase test combining low-speed centrifugation and the use of a group specific anti-VP1 monoclonal antibody (5-D8/1, Dako) was developed. This rapid culture assay (RCA) was compared blindly to a conventional culture assay (CCA) on a panel of 180 children's stool specimens received for routine diagnosis of enterovirus infection. The same cell lines (human embryonic fibroblasts and KB continuous cell line) were used in both tests. Discrepancies in results were analysed by a PCR technique with primers located in a conserved part of the 5′ non-coding region of the enterovirus genome. Fourteen specimens were positive and 158 were negative with both tests. Four samples were positive with the RCA yet negative with the CCA and 3 others showed the opposite pattern; an additional sample positive by RCA was uninterpretable by CCA due to bacterial contamination. Subsequent PCR testing of these 8 samples showed no discrepancies; all were positive. Using CCA as the reference, the sensitivity and specificity of RCA were 77.8 and 98% respectively. Kinetic studies using enterovirus isolates demonstrated that RCA was much more sensitive than CCA during the first three days of culture. These results further suggested that RCA sensitivity could be improved by a factor of at least 10 times by prolonging the incubation period by 24 hr. With this change, the RCA assay described below is suggested as a rapid alternative to CCA for the routine diagnosis of enterovirus infection in stool specimens. When an identification at the serotype level is required, samples found positive using RCA could then be subjected to CCA. ***J. Med. Virol. 54:204–209, 1998.*** © 1998 Wiley-Liss, Inc.

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INTRODUCTION

Enterovirus is a large genus of RNA viruses, including at least 65 serotypes responsible for acute and chronic diseases in man [Melnick, 1991; Minor et al., 1995]. Due to this antigenic diversity, the diagnosis of enterovirus infection has been mainly based on conventional cell culture using different cell lines to increase the sensitivity of the method [Menegus, 1985]. Molecular techniques based on the amplification of conserved parts of the enterovirus genome (especially in the 5′ non-coding region) have demonstrated recently their superiority over cell culture in terms of sensitivity and rapidity for the diagnosis of enteroviral meningitis from cerebrospinal fluid [Abzug et al., 1995; Lina et al., 1996; Rotbart et al., 1994; Schlesinger et al., 1994; Yerly et al., 1996]. However, these techniques still lack standardization for heterogeneous biological specimens such as stool or throat specimens. PCR inhibitors may cause false negative results and a positive result does not necessarily indicate the presence of viable virus.

A cost-effective alternative to PCR for the diagnosis of enterovirus infection in peripheral samples could be the use of a rapid cell culture assay followed by the detection of enterovirus cytopathic effect (CPE) using a group-specific monoclonal antibody, as has been described recently [Klespies et al., 1996]. The results are described of a prospective study comparing rapid culture assay (RCA) and conventional culture assay (CCA)

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for the detection of enterovirus in stool specimens received in our laboratory on a routine basis. Monoclonal antibody 5-D8/1 (Dako, Trappes, France) directed towards a highly conserved epitope in VP1 and which has been shown to recognize most enterovirus serotypes detected by cell culture [Trabelsi et al., 1995] was used.

MATERIALS AND METHODS

Stool Specimens

A total of 180 unselected stool specimens obtained from children and neonates sent to the laboratory for viral culture between April and June 1996 on a routine basis were included prospectively in the study. A small volume (0.5–2 g) of stools was diluted in 20 ml of culture medium composed of basal medium of Eagle (BME) with 1% Ultroser® G (GIBCO BRL, Cergy-Pontoise, France), antibiotics and amphotericin B, frozen overnight at −20°C and centrifuged 25 min at 6,000 × *g;* the supernatant constituted the sample for the two cell culture assays and the PCR assay where necessary.

Cells and Viruses

Human embryonic lung fibroblasts (HEL line, Bioproducts, Gagny, France) and KB cells were used in both culture tests. Cell tubes (85,000 and 50,000 cells per tube for HEL and KB cells respectively) and 96-well microplates (10,000 cells per well) were used within 4 days after seeding with freshly trypsinized cells.

Four clinical isolates belonging to different enterovirus serotypes (coxsackievirus (CV) B3 and B4, echovirus (EV) types 11 and 25) were used in the kinetic experiments carried out to evaluate the sensitivity of the rapid culture.

Conventional Cell Assay (CCA)

Conventional culture of stool specimens was undertaken following standard procedures [Pozzetto et al., 1989]. Each specimen was used to inoculate two tubes for each of the two cell lines. The tubes were examined microscopically at regular intervals up to 12 days after inoculation. Systematic passages were done when a toxic effect was noted. In the case of bacterial or fungal contamination, the stool extract was treated again with anti-bacterial or anti-fungal agents and retested using the CCA. When an enteroviral CPE was observed, the culture was passaged onto fresh cells. The enterovirus infection was confirmed by an immunofluorescence test using the enterovirus monoclonal antibody 5-D8/1 as previously described [Trabelsi et al., 1995]. The culture supernatant was then titrated on 96-well microplates seeded with the same cell line as the one on which the CPE was detected. About 100 50% tissue culture infectious doses ($TCID_{50}$) were used for neutralization testing with intersecting pools of hyperimmune horse sera (Statens Serum Institute, Copenhagen, Denmark), following Lim–Benyesh-Melnick's schema [Lim and Benyesh-Melnick, 1960; Melnick and Wimberly, 1985]. Further identifications were made by neutralization with a specific monovalent polyclonal antiserum.

Rapid Culture Assay (RCA)

This method consisted of a microplate centrifugation followed by an 18 hr culture and immunoperoxidase reaction as described previously for the detection of cytomegalovirus in urine specimens [Pozzetto et al., 1990]. Stool extracts, tested both undiluted and diluted 1:2, 1:4 and 1:8 in the culture medium, were inoculated onto microplates seeded with HEL or KB cells (two wells per dilution and per cell line; 100 μl per well). In each experiment, a negative control (culture medium) and a positive control (clinical isolates of diluted CVA-9 and CVB-3 for HEL and KB cells respectively) were tested in parallel. After a 45 min centrifugation at 3,000 × *g* and 30°C, the plates were emptied, refilled with fresh medium (200 μl per well) and placed at 37°C in a CO_2 incubator for about 18 hr. The culture medium was then removed and the cells were fixed with 90% (v/v) cold acetone in deionized water (200 μl per well) and treated with the 5-D8/1 monoclonal antibody diluted 1:40 in deionized water (50 μl per well) for 30 min at 37°C. After 4 washing steps with phosphate buffer saline (PBS), a sheep anti-mouse peroxidase-labelled antibody (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) diluted 1:200 in deionized water was added (50 μl per well) for 30 min at 37°C. After repeat washing, the cells were treated with peroxidase substrate (diaminobenzidine (Sigma, Saint Quentin Fallavier, France) 0.05% (w/v) and H_2O_2 0.55% (v/v) in 0.1 M Tris buffer) (50 μl per well) for 30 min at room temperature in the dark and the reaction was stopped by washing with PBS. Screening was carried out using 40 × magnification and either 100 or 250 × magnification for confirmation of positive results. A well containing at least one focus of cells with dark-brown cytoplasmic coloration (Fig.1) was considered positive.

PCR Assay

The PCR assay used in this study was a commercially-available technique (Amplicor Enterovirus, Produits Roche, Neuilly sur Seine, France). The RNA extraction from stool supernatants used TRI-REAGENT® (Sigma) consisting of a mixture of guanidium and phenol, following the one-step procedure of Chomczynski and Sacchi [1987]. One hundred μl of stool extract was mixed with 1 ml of TRI-REAGENT®. After a 10 min centrifugation at 12,000 × *g* and 4°C to remove insoluble material, the supernatant was mixed with 10% chloroform (v/v). After a 15 min centrifugation under the same conditions, the aqueous phase was precipitated overnight using isopropanol at −20°C. After another 15 min centrifugation, the pellet was washed twice in 75% ethanol, dried and resuspended in 50 μl of the PCR buffer available in the commercial kit. With the exception of the extraction step, the PCR assay was performed according to the manufacturer's instructions and as previously reported [Lina et al., 1996]. The results were scored as positive when the optical density at 450 nm was above 0.35.

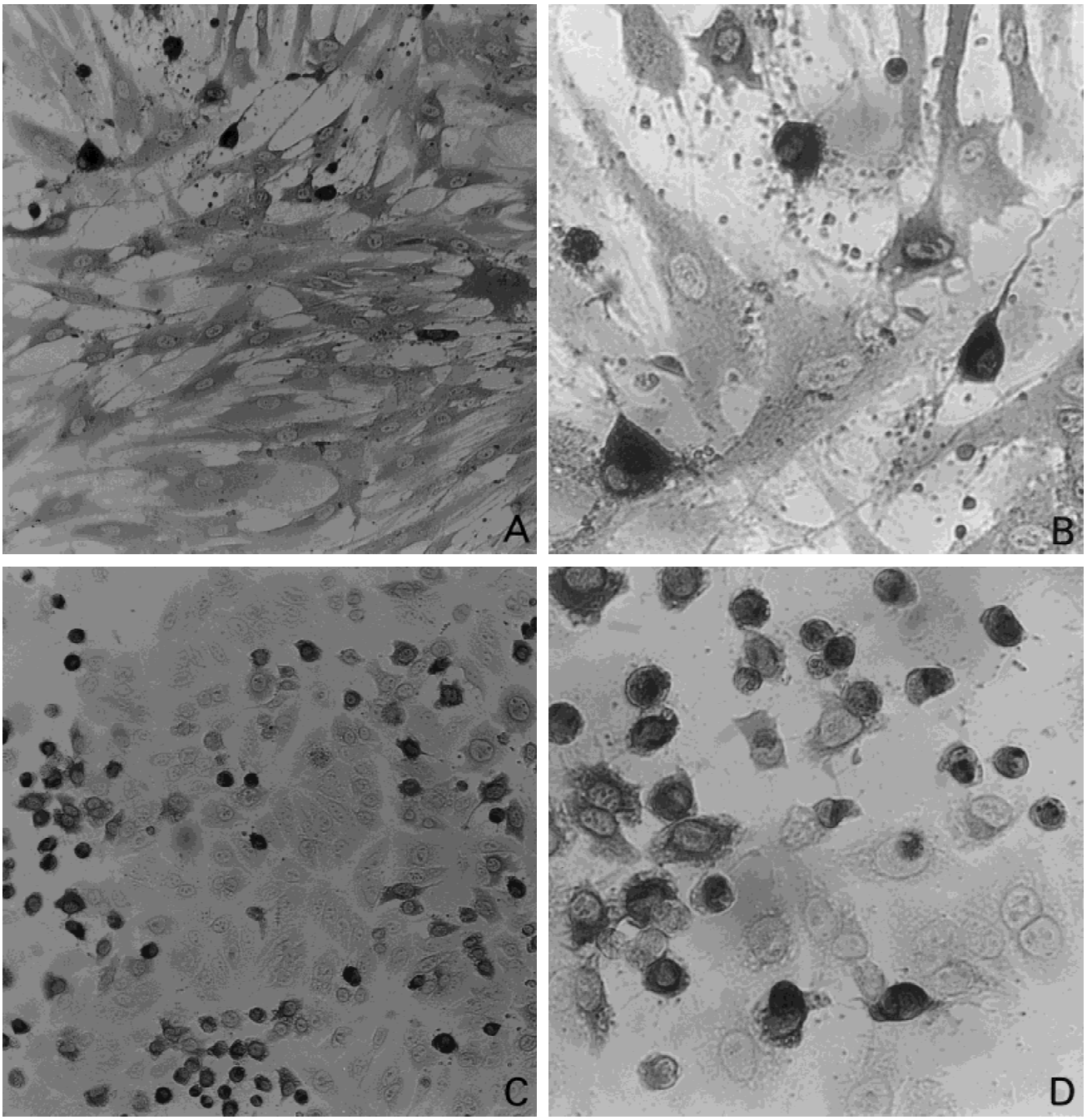

Fig. 1. Typical enteroviral cytopathic effects by rapid culture assay using immunoperoxidase staining on HEL (panels A and B) and KB cells (panels C and D) at 100× (panels A and C) and 250× (panels B and D) magnification.

Design of the Comparative Study

The study was conducted prospectively on 180 unselected stool specimens of paediatric origin. CCA was performed as routine; the microscopic examination was done by one of the team. RCA was performed on the same stool extracts kept frozen at −20°C within one month. The microplate reading was done in a blind fashion with regard to the CCA results by at least two of us. When discrepancies between the two techniques were noted, the corresponding stool extract (kept frozen at −20°C) was retested by both culture techniques and by PCR.

Evaluation of RCA Sensitivity

Four clinical isolates of enterovirus belonging to different serotypes (CV-B3, CV-B4, EV-11 and EV-30) were used to evaluate the sensitivity of the RCA compared to the CCA. Each strain was tested in 96-well microplates at 6 serial ten-fold dilutions from 10^{-1} to 10^{-7}, using 6 replicates per dilution. The microplates

TABLE I. Comparison of Conventional Cell Assay (CCA) and Rapid Cell Assay (RCA) for the Detection of Enterovirus in Stool Specimens

Results of detection method		No. (%) of stool specimens
CCA	RCA	
Negative	Negative	150 (83.3)
Negative	Positive	3 (1.7)
Bacterial contamination	Negative	8 (4.4)
Bacterial contamination	Positive	1 (0.5)
Positive	Negative	4 (2.2)
Positive	Positive	14 (7.8)

used for the CCA were read successively after 18, 30, 42 and 68 hr of incubation; the final titre, used as reference, was determined after 96 hr incubation. At the same time, 4 identical microplates were prepared for each virus serotype and tested by immunoperoxidase staining after 18, 30, 42 and 68 hr incubation respectively. CVB3 and CVB4 were tested on KB cells and EV-11 and EV-30 on HEL cells.

RESULTS

One hundred and eighty stool specimens were tested using both assay techniques (Table I); 164 specimens gave concordant results (150 negative and 14 positive). Nine samples gave uninterpretable results by CCA due to bacterial contamination; one of these specimens was found positive by RCA (Table I). Three samples were positive by RCA yet negative by CCA and four others showed the opposite pattern (Table I). The four latter samples corresponded to stools exhibiting low viral titres using CCA ($\leq 10^3$ $TCID_{50}$/100 µl); in addition, three of them were toxic at 1:1 (2 samples) and 1:2 dilutions (one sample) with the RCA. Using CCA as the gold standard, RCA exhibited a sensitivity of 77.8% and a specificity of 98%.

Using CCA, 7 samples showed the presence of an adenovirus but were all negative for enterovirus. The RCA however, detected an enterovirus in one of these samples. The mean time necessary to detect and confirm the presence of an enterovirus was 11.5 ± 5 days for the 18 specimens found positive using CCA. The serotypes of these 18 enteroviruses were as follows: EV-11 in 10 cases, EV-30 in 2 cases, CVB-3 in 2 cases and CVB-1 in 1 case. Three strains were not typeable by intersecting pools of antisera (Table II). The 4 samples giving positive results only with the RCA could not be submitted to standard identification.

All discordant samples were subjected to the Amplicor Enterovirus PCR assay with the slight modifications described above. As indicated in Table II, they were all confirmed positive by this technique. The specificity of the method was verified using controls (water and non-infected stool specimens which were found negative for the presence of enterovirus genome). Thus, CCA or RCA positive specimens which were also found positive using PCR were considered to be true-positive specimens.

As for cell sensitivity, all but 3 of the positive specimens cultured on a single cell line. For all samples found positive by both tests (including the 3 samples positive for the two cell lines), the enterovirus CPE was detected on the same cell line(s).

TABLE II. Features of the 22 Stool Specimens Found Positive for Enterovirus Detection by Conventional Cell Assay (CCA) and/or Rapid Cell Assay (RCA)

Results of detection method		Enterovirus serotype[a]	No. samples	PCR results
CCA	RCA			
+	+	EV-11	8	ND[b]
+	+	EV-30	2	ND
+	+	CVB-1	1	ND
+	+	CVB-3	1	ND
+	+	Not typeable	2	ND
+	−	EV-11	2	+
+	−	CVB-3	1	+
+	−	Not typeable	1	+
−	+	NA[c]	3	+
Contaminated[d]	+	NA	1	+

[a]EV: echovirus; CVB: coxsackievirus B.
[b]ND: not done.
[c]NA: not available.
[d]Bacterial contamination.

The toxicity of stool specimens in RCA, defined as the presence of a toxic effect in both wells of a duplicate assay for the same dilution, was as follows: for HEL cells, 44.4, 17.8 and 7.8% of samples were toxic at dilutions 1:1, 1:2 and 1:4 respectively; for KB cells, the figures were 28.9, 13.3 and 3.9% for the same dilutions respectively. At dilution 1:8, no sample exhibited a toxic effect for both cell lines. Table III illustrates the relation between toxicity and the threshold of positivity according to sample dilution for the 14 stool specimens found to be positive for enterovirus with the RCA. These results suggest that dilution of samples probably impairs the sensitivity of the assay.

In order to obtain more details on the sensitivity of the RCA as compared to CCA, serial titration experiments were carried out on 4 clinical strains belonging to different enterovirus serotypes, as described above. Table IV summarizes the results of these kinetic experiments. For all 4 strains, sensitivity of the RCA was dramatically higher than that of the CCA after 18 and 30 hr incubation; after 68 hr, the sensitivity of the two tests was roughly equivalent. These results also show that the sensitivity of RCA could be improved by a factor of more than ten times when prolonging the incubation by 24 hr before immunoperoxidase staining (Table IV).

DISCUSSION

Techniques using spin amplification and identification by a specific monoclonal antibody have been shown to shorten significantly the detection time of many viruses in cell culture, including cytomegalovirus [Agha et al., 1988; Alpert et al., 1985; Gleaves et al., 1984; Pozzetto et al., 1990], adenovirus [August and Warford, 1987; Trabelsi et al., 1992] and respiratory viruses [Al-Mulla et al., 1994; Olsen et al., 1993], with excellent

TABLE III. Comparison of Toxicity and Threshold of Positivity According to Sample Dilution on 14 Stool Specimens Found Positive for the Presence of Enterovirus by RCA

	HEL cells (8 positive samples)		KB cells (12 positive samples)	
Sample dilution	Toxic effect (No. samples)	First + result (No. samples)	Toxic effect (No. samples)	First + result (No. samples)
1:1	6	2	3	4
1:2	1	2	1	5
1:4	0	1	0	1
1:8	0	3	0	2

TABLE IV. Comparison of the Sensitivity of CCA and RCA on 4 Clinical Strains of Enterovirus at Different Times of Incubation

	Reduction in titre expressed as $\log_{10}$[2]							
	CV-B3		CV-B4		EV-11		EV-30	
Time of incubation[1]	CCA	RCA	CCA	RCA	CCA	RCA	CCA	RCA
18 h	4.2	1.7	4.7	2.2	3.3	2.3	3.0	1.9
30 h	3.5	0.7	3.5	1.5	2.3	0.5	1.9	0.8
42 h	2.2	0.5	2.4	1.2	1.4	0.3	0.9	0.4
68 h	0.7	0	0.7	0	0.3	0.4	0.4	0.2

Serial ten-fold dilutions of each strain were tested in 96-well microplates seeded with cells (six replicates by dilution). The detailed procedure is reported in the text.
[1]This time corresponds to the incubation period before microplate reading for CCA and before acetone fixation and immunological staining for RCA.
[2]The virus titres used as reference were those obtained by CCA after 96 hr of incubation ($10^{6.7}$ for CV-B3, 10^{7} for CV-B4, $10^{5.8}$ for EV-11 and $10^{5.4}$ for EV-30).

sensitivity and specificity. In this study, the combined effect of sample centrifugation and immunological staining is probably responsible for the dramatic increase in sensitivity of RCA compared to CCA in the first three days of virus replication, as exemplified by the data in Table IV.

In another study suggesting the same approach for the diagnosis of enterovirus infection in various biological specimens, the investigators used a 72 hr spin-amplified shell vial indirect immunofluorescence assay [Klespies et al., 1996]. Compared to this rapid culture technique, the method described above involving microplate culture and immunoperoxidase identification, is easier to perform; the washing steps can be automated, cover slips are not required avoiding the risk of mismatching samples, a large number of specimens can be processed simultaneously and microscopic examination ensures clear-cut results (Fig. 1).

In our study, the mean detection time of enterovirus-infected specimens was reduced from 11.5 days using CCA to less than two days with RCA. The availability of results in a short time is very useful for clinical purposes: in the case of a positive result, useless treatments such as antibiotics or antiherpetic agents can be ceased and with negative results, further investigations can be suggested rapidly. RCA is also less susceptible to bacterial or fungal contamination than CCA (Table I) and, in the case of dual infection (as exemplified in this study by one specimen positive for both adenovirus and enterovirus), allows the detection of the enterovirus CPE, which is not the case with the CCA. From an economic viewpoint, the cost of the monoclonal antibody is counterbalanced by a dramatic reduction in laboratory time.

Sample toxicity is a major problem with stools in cell culture. When toxicity occurs in CCA, a subculture must be done. In order to overcome this difficulty, specimen dilutions were performed for the RCA. A progressive disappearance of the toxic effect was noted in parallel with the dilution of stool extracts; however, this sample dilution does impair the sensitivity of the assay (Table III), as illustrated by four specimens found negative by RCA but positive using CCA and PCR. The kinetic experiments on clinical strains (Table IV) have suggested that the sensitivity of RCA could be improved significantly by prolonging the incubation period by 24 hr. Another limitation of this RCA lies in the fact that the identification of enteroviruses at serotype level is not possible. If it is not relevant for clinical purposes, it may be critical for epidemiological studies. It is always possible to test RCA-positive samples using CCA whenever necessary.

Although these results are encouraging, further studies are required to confirm that RCA can be substituted for CCA without loss of sensitivity for routine detection of enteroviruses by cell culture. The results suggest that the contact between cells and samples could be extended to 42 hr (the test proposed by Klespies and colleagues [1996] was carried out over 72 hr). Although the 5-D8/1 monoclonal antibody is able to detect a wide range of enterovirus serotypes without cross-reactivities [Trabelsi et al., 1995; Yousef et al., 1987], some variant strains can escape antibody recognition. Using a mixture of several monoclonal antibodies could further improve the sensitivity of the assay. Another limitation of the test described herein is the fact that it is restricted to the detection of enteroviruses, excluding other viruses such as adenoviruses

usually detected by cell culture in stool specimens. This disadvantage could be avoided by the combination of two RCA on the same samples using enterovirus and adenovirus group-specific monoclonal antibodies; in fact, we have already shown that a similar technique can be applied to adenoviruses [Trabelsi et al., 1992]. Further such studies are under development in our laboratory.

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